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Induction of TIMP-1 expression in rat hepatic stellate cells and hepatocytes: a new role for homocysteine in liver fibrosis

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Abstract

Elevated plasma levels of homocysteine have been shown to interfere with normal cell function in a variety of tissues and organs, such as the vascular wall and the liver. However, the molecular mechanisms behind homocysteine effects are not completely understood. In order to better characterize the cellular effects of homocysteine, we have searched for changes in gene expression induced by this amino acid. Our results show that homocysteine is able to induce the expression and synthesis of the tissue inhibitor of metalloproteinases-1 (TIMP-1) in a variety of cell types ranging from vascular smooth muscle cells to hepatocytes, HepG2 cells and hepatic stellate cells. In this latter cell type, homocysteine also stimulated α1(I) procollagen mRNA expression. TIMP-1 induction by homocysteine appears to be mediated by its thiol group. Additionally, we demonstrate that homocysteine is able to promote activating protein-1 (AP-1) binding activity, which has been shown to be critical for TIMP-1 induction. Our findings suggest that homocysteine may alter extracellular matrix homeostasis on diverse tissular backgrounds besides the vascular wall. The liver could be considered as another target for such action of homocysteine. Consequently, the elevated plasma levels of this amino acid found in different pathological or nutritional circumstances may cooperate with other agents, such as ethanol, in the onset of liver fibrosis.

Keywords: Hyperhomocysteinemia; Extracellular matrix; Liver; Fibrosis; Risk factor

1. Introduction

Elevated blood levels of homocysteine, an intermediate metabolite of methionine, have been identified as an independent risk factor for peripheral vascular disease and arteriosclerosis (reviewed in [1]). Hyperhomocysteinemia is caused by genetic factors, such as cystathionine β-synthase deficiency [2], and acquired conditions including deficient intake of vitamin B_6, B_{12} and folate [3], required for homocysteine metabolism. Additionally, impaired liver func-

Abbreviations: VSMC, vascular smooth muscle cell; TIMP, tissue inhibitor of metalloproteinases; HSC, hepatic stellate cell; ECM, extracellular matrix; MMP, matrix metalloproteinase; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; AP-1, activating protein 1; DTT, dithiothreitol

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tion has been associated with elevated plasma levels of this amino acid [4–7]. This last situation probably stems from the central role of the liver in the metabolism of methionine and subsequently in the catabolism of homocysteine [8,9].

Regarding the pathological mechanisms behind homocysteine-induced cellular toxicity it has been observed that different cell types can be targeted by this amino acid. Endothelial injury resulting in a deranged NO production, and consequently an impaired platelet modulating activity has been clearly established [10,11]. Along with this effect on the vessel wall, homocysteine is able to promote DNA synthesis and to enhance collagen production in vascular smooth muscle cells (VSMCs) [12–15]. In addition, it has been recently demonstrated that hepatic cells produce and secrete more cholesterol in response to homocysteine [16]. Taken together, these observations suggest that homocysteine exerts its effects on a variety of cellular backgrounds, and thus the deleterious consequences of hyperhomocysteinemia may spread over different tissues and organs.

In order to gain more insight into the molecular mechanisms responsible for the interference of homocysteine in normal cell function, we have searched for genes whose expression could be altered in the presence of physiopathological concentrations of this amino acid. In this report, we describe the induction by homocysteine of the tissue inhibitor of metalloproteinases-1 (TIMP-1) [17]. This effect of homocysteine, that we initially observed in VSMCs in culture, could be demonstrated in other cell types, such as hepatic stellate cells (HSC) and hepatocytes. TIMP-1 is a low molecular weight glycoprotein that plays a key role in the regulation of extracellular matrix (ECM) accumulation through its ability to inhibit matrix metalloproteinases (MMPs). When TIMP-1 is produced in excess the net MMP activity is reduced. This situation has been proposed to be of importance in the development of liver fibrosis [18]. High TIMP-1 levels have been observed in diseased human liver [19,20] and experimental models of liver injury [20–22], situations in which elevated plasma concentrations of homocysteine occur [4–7]. Our results showing a direct effect of homocysteine on the induction of TIMP-1 and α1(I) procollagen expression suggest that elevated levels of this amino acid may participate in the pathogenesis of liver fibrosis.

2. Materials and methods

2.1. Materials

D,L-Homocysteine was from Sigma (St. Louis, MO). Cell culture media, fetal bovine serum and antibiotics were from Gibco-BRL (Paisley, UK). Anti TIMP-1 monoclonal antibody was from Calbiochem (San Diego, CA). Radioactive isotopes were purchased from Amersham (Little Chalfont, UK). All other reagents were of the best quality commercially available.

2.2. Methods

2.2.1. Cell culture

The hepatic stellate cells (HSC) clone CFSC-2G derived from CCL_4-treated rats [23], kindly provided by Dr. Marcos Rojkind, was maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum and non-essential amino acids. This cell line has a similar phenotype to that of freshly isolated HSC. Human hepatoma cell line HepG2 was kept in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum. In all the cases, media was supplemented with 2 mmol/l glutamine, 100 U/ml penicillin and 50 mg/ml streptomycin sulfate. All experiments with these cells were performed in serum-free medium supplemented with 0.2% bovine serum albumin fraction V (Sigma).

Human VSMCs were isolated from mammary artery using the explant method, accordingly with previously described procedures [24]. Porcine VSMCs were isolated from aorta of Yucatan mini-pigs, in sterile conditions, immediately after animal sacrifice. Immunohistochemical characterization of VSMC isolates was performed on primary cultures, using specific antibodies against smooth muscle specific α-actin (Dako, High Wycombe, UK). VSMCs were grown in DMEM supplemented with antibiotics as described above plus fungizone (Gibco-BRL) and 10% fetal calf serum. Experiments described herein used different isolates of VSMC between passages 3 and 6. Subconfluent cultures of VSMCs were washed twice with DMEM and rendered quiescent by serum deprivation and maintenance in serum-free medium (DMEM/F12, Gibco-BRL) containing insulin, transferrin and sodium selenite (ITS-x, Gibco-BRL) for

48 h before the beginning of the experimental procedures. Incubations with D,L-homocysteine were carried out in serum-free DMEM medium.

Rat hepatocytes were isolated and cultured as previously described [25]. Cultures were kept in 0.5% fetal calf serum for 16 h before treatments, which were carried out in the absence of serum.

All cells were maintained at 37°C in a humidified incubator containing 21% oxygen and 5% carbon dioxide in air.

The investigation was performed in accordance with the European Community guidelines for animal ethical care and use of laboratory animals (Directive 86/609), and fulfilled the ethical requirements of this University.

2.2.2. Differential display analysis

Total cellular RNA was isolated by extraction with guanidinium thiocyanate followed by centrifugation in a cesium chloride solution as previously described [26]. A 25-μg amount of total RNA was incubated for 30 min at 37°C with 10 U of RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany) in 10 mmol/l Tris HCl pH 7.5, 10 mmol/l $MgCl_2$, then samples were phenol:chloroform extracted and ethanol precipitated in the presence of 0.3 mol/l sodium acetate. RNA was redissolved in sterile nuclease-free water.

Differential display was performed using oligo(dT) anchored primers [26,27] with the Hieroglyph mRNA Profile Kit (Genomyx, Beckman Instruments, Fullerton, CA) following the manufacturer's instructions with some modifications. First strand cDNA synthesis was performed with 2 μl of DNase-treated total RNA (0.1 μg/μl) and 2 μl of oligo(dT) anchored primer (2 μmol/l) using First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Barcelona, Spain) in a Perkin-Elmer GeneAmp 2400 thermal cycler at 25°C (10 min), 42°C (60 min) and 70°C (15 min). The polymerase chain reaction (PCR) started by adding 2 μl of the cDNA solution to a mixture containing 9.95 μl of sterile nuclease-free water, 1.2 μl $MgCl_2$ (25 mmol/l), 1.6 μl dNTP mix (1:1:1:1) (250 μmol/l ea) (Boehringer Mannheim), 2 μl 5′-arbitrary primer (2 μmol/l), 2 μl 3′-oligo(dT) anchored primer (2 μmol/l), 2 μl AmpliTaq Buffer (10×), 0.2 μl AmpliTaq enzyme (5 U/μl) (Perkin-Elmer, Branchburg, NJ), and 0.25 μl [α-^{33}P]dATP (10 μCi/μl). Thermal cycling parameters using GeneAmp 2400 thermal cycler were as follows: 92°C (2 min), 4 cycles at 92°C (15 s), 46°C (30 s), and 72°C (2 min), 30 cycles at 92°C (15 s), 60°C (30 s), and 72°C (2 min), and an additional final extension step at 72°C for 7 min. Reactions were performed with each cDNA solution in duplicate. Control reactions were set using sterile nuclease-free water or each DNase-treated RNA instead of the cDNA solution.

Following differential display PCR, radiolabeled cDNA fragments were electrophoretically separated on 4.5% polyacrylamide gels under denaturing conditions in a Genomyx LR DNA sequencer (Genomyx, Beckman). Gels were dried and exposed to produce an autoradiograph. Bands of interest were excised from the gel, and the gels slides were placed directly into PCR tubes and covered with 40 μl of PCR mix (24.4 μl sterile nuclease-free water, 3.2 μl dNTP mix, 4 μl T7 promoter 22-mer primer (2 μmol/l), 4 μl M13 reverse 24-mer primer (2 μmol/l), 2.4 μl $MgCl_2$ (25 mmol/l), 4 μl AmpliTaq PCR Buffer (10×), and 0.4 μl AmpliTaq enzyme (5 U/μl)). PCR was performed as follows: 95°C (2 min), 4 cycles at 92°C (15 s), 50°C (30 s), and 72°C (2 min), 30 cycles at 92°C (15 s), 60°C (30 s), and 72°C (2 min), and an additional extension step at 72°C for 7 min. Amplified DNA fragments were cloned into the plasmid vector pCR2:1-TOPO using TOPO-TA Cloning Kit (Invitrogen, Leek, The Netherlands) and sequenced in both directions using M13 reverse (−24) primer and M1 forward (−20) primer. Nucleotide sequence homology search analysis of the EMBL [28] and GeneBank [29] databases were performed using the program FASTA [30].

2.2.3. RNA isolation and Northern blot analysis

Total RNA from the different cell lines used was isolated by the guanidinium thiocyanate method [31]. Aliquots (20 μg) of total RNA were size-fractionated by electrophoresis in a 1% agarose gel under denaturing conditions. RNAs were then blotted and fixed to Nytran membranes (Schleicher and Schuell, Keene, NH). Prehybridization and hybridization were performed as described previously [32]. Probes used were the isolated clone from differential display (clone 3.3) and a 1.6-kb cDNA fragment of procollagen α1 [33]. Equal loading of the gels was assessed

by hybridization with a probe specific for β-actin. The probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by random priming using the Megaprime DNA labeling system (Amersham). Specific activity was usually approximately 5×10^8 cpm/μg of DNA. Quantitation was performed by scanning densitometry of the X-ray films.

2.2.4. Immunoblot analysis

Conditioned media from equal number of HepG2 cells were collected after treatments and concentrated 10-fold using Centricon 3 concentrators (Amicon, Beverly, MA). Samples were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell). Immunodetection of TIMP-1 was performed using a monoclonal anti TIMP-1 antiserum (Calbiochem) and a horseradish peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminescence according to the manufacturer's instructions (Dupont, Boston, MA).

2.2.5. Nuclear protein extraction and electrophoretic mobility shift assays (EMSA)

Rat hepatocytes were incubated in low serum (0.5%) for 16 h before treatment. Nuclear proteins from control and treated cells were isolated following the method described by Schreiber et al. [34]. An oligonucleotide containing the AP-1 consensus sequence (5′-TTCCGGGTGACTCATCAAGCG-3′) (Promega), was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The reaction mixture (25 μl final volume) containing 40% glycerol, 1 μg of dIdC, 10 mmol/l EDTA, 20 mmol/l DTT, 100 mmol/l Tris HCl buffer pH 7.5, 10 000 cpm of labeled oligonucleotide and 10 μg of nuclear extract was incubated for 30 min at room temperature. DNA–protein complexes were separated from unbound probe by electrophoresis on a 5% polyacrylamide gel. Complexes formed were identified by autoradiography of the dried gels. For competition studies, the reaction was carried out as described above, but in the presence of the indicated concentration of unlabeled probe.

2.2.6. Statistics

The data shown are the means ± S.E.M. of at least three independent experiments. Statistical significance was estimated with Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

3. Results

Differential display analysis was carried out on human VSMCs isolated from mammary artery. Cells were made quiescent by serum deprivation and then treated with 100 μmol/l of homocysteine, a concentration compatible with intermediate hyperhomocysteinemia [1], for 24 h in DMEM medium. After this treatment total RNA was isolated and the differential display analysis was performed as described

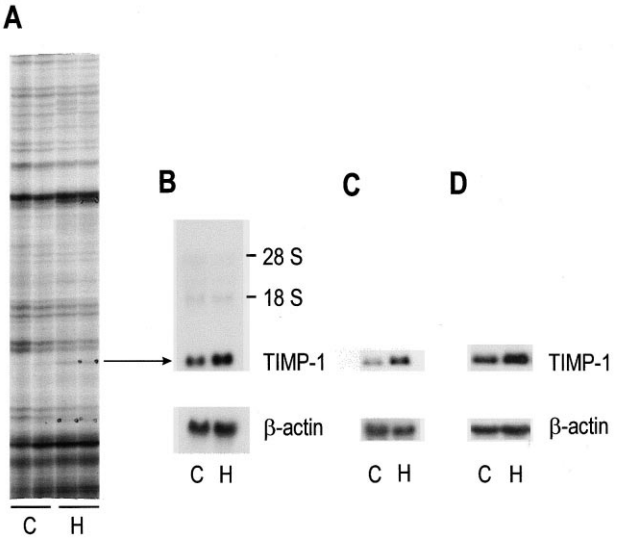

Fig. 1. Detection of differential gene expression induced by homocysteine treatment in human VSMCs by differential mRNA display analysis. (A) Sequencing gel electrophoresis of PCR amplified cDNAs performed in duplicates, from control (C) and homocysteine (100 μmol/l, 24 h) (H) treated cells. A differentially displayed fragment (arrow) was detected, isolated, sequenced and identified as a 0.6-kb fragment of TIMP-1 cDNA. (B) Northern blot analysis of total RNA from control (C) and homocysteine treated (H) human VSMCs performed with TIMP-1 cDNA fragment. This assay confirmed its differential expression between control and treated cells, detecting a 0.9-kb transcript which was induced upon treatment. (C) Northern blot analysis of total RNA from control (C) and homocysteine (100 μmol/l for 24 h) treated (H) pig VSMCs performed with TIMP-1 cDNA fragment. (D) Northern blot analysis of total RNA from control (C) and homocysteine (H) (150 μmol/l) treated rat HSCs. Blots were sequentially probed with β-actin as loading control. The migration of ribosomal markers is indicated. Representative blots are shown.

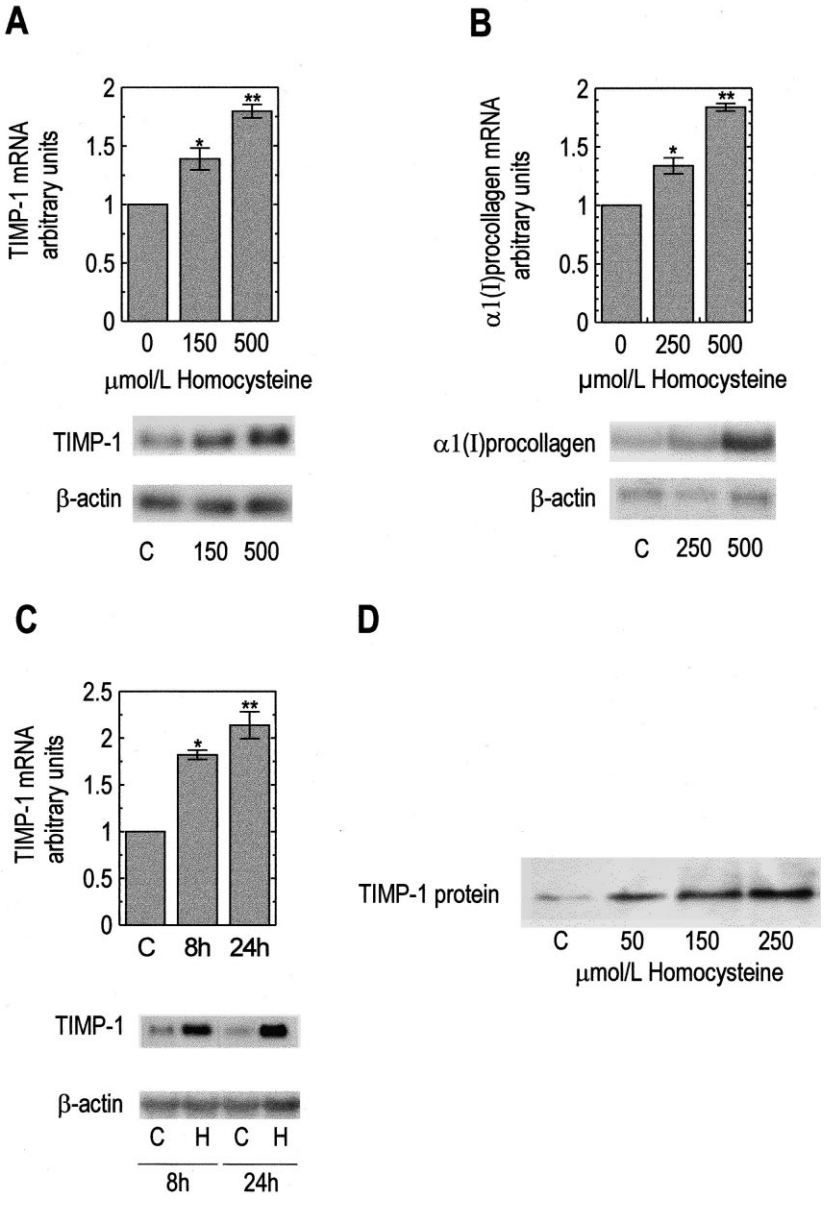

Fig. 2. Induction of TIMP-1 and α1(I) procollagen expression by homocysteine. (A) Rat HSCs were treated for 4 h with different doses of homocysteine and TIMP-1 mRNA levels were determined by Northern blotting. (B) Dose-dependent induction of α1(I) procollagen mRNA in HSCs cells treated for 4 h with increasing concentrations of homocysteine assayed by Northern blotting. (C) Effect of homocysteine (500 μmol/l) on TIMP-1 mRNA expression in cultured rat hepatocytes after different incubation times. Blots were probed with β-actin for loading control. Representative blots are shown. (D) Detection of TIMP-1 immunoreactivity in the conditioned media of human hepatoma HepG2 cells treated with different concentrations of homocysteine for 24 h. Conditioned media were concentrated and analyzed by Western blotting, a single band of about 29 kDa was detected which was significantly increased upon treatment with respect to the untreated control cells (C). A representative blot is shown. Asterisks denote statistical significance (*statistically different ($P < 0.05$) to control value, **statistically different ($P < 0.05$) to control value and previous dose/time point).

in Section 2. Several bands were differentially expressed in cells treated with homocysteine as compared with control cultures. One of the bands selected for analysis, whose expression was induced in homocysteine-treated cells (Fig. 1A), was excised from the gel, amplified and sequenced. This clone of about 0.6 kb (termed clone 3.3) had a sequence 100% identical to part of the human TIMP-1 cDNA

(nucleotides 165–615) [35] and could hybridize a 0.9-kb mRNA in total cellular RNA from human VSMCs (Fig. 1B). The band detected in Northern blot by clone 3.3 corresponded in size to that reported for TIMP-1 transcript in other human cell lines [36,37], and was induced 2-fold upon treatment with homocysteine, thus confirming the induction detected in the differential display gel. The effect of homocysteine on TIMP-1 expression was also tested on VSMCs obtained from pig aorta. As shown in Fig. 1C, incubation of these cells with 100 μmol/l of homocysteine for 24 h resulted in a 2.5-fold induction in TIMP-1 mRNA, demonstrating that this effect was not species specific. Given the central role played by HSC in liver matrix biology [38,39], we next examined TIMP-1 levels in response to homocysteine in a rat HSC line. As shown in Fig. 1D, treatment of HSC with 150 μmol/l of homocysteine for 4 h resulted in a 1.5-fold increase in TIMP-1 mRNA levels. HSC response to homocysteine was maximal after 4 h of treatment and dose-dependency was studied at this time point as shown in Fig. 2A. Homocysteine has been reported to induce the expression of α1(I) procollagen mRNA and to promote collagen synthesis in VSMCs in culture [13,14]. We have examined whether homocysteine could elicit a similar response on HSCs. Treatment of HSCs with increasing concentrations of homocysteine resulted in a dose-dependent increase in α1(I) procollagen mRNA, reproducing in this cell type the response described by others in VSMCs (Fig. 2B).

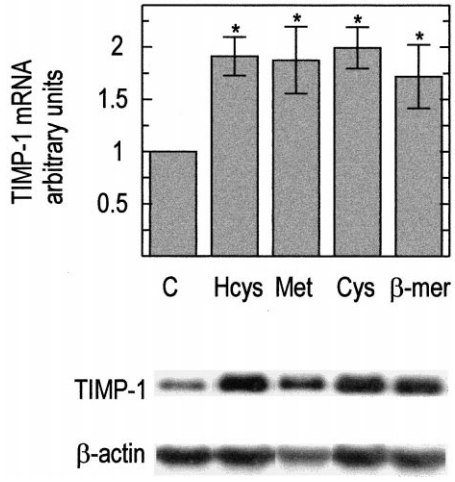

Fig. 3. TIMP-1 induction in HSCs by thiol-bearing agents and methionine. HSCs were treated with 500 μmol/l of homocysteine, methionine, cysteine or β-mercaptoethanol for 4 h, TIMP-1 mRNA levels were assayed by Northern blotting. Blots were probed with β-acting for loading control, a representative blot is shown. Asterisks denote statistical significance ($P < 0.05$).

Another important source of TIMP-1 within the liver tissue is the parenchymal cell. TIMP-1 is induced in hepatocytes in diseased human liver [40] and in different models of liver injury as well as in rat hepatocytes in primary culture by cytokines [41]. These observations prompted us to examine whether homocysteine could promote a similar response in isolated rat hepatocytes. Treatment of cultured rat hepatocytes with different concentrations of homocysteine resulted in the induction of TIMP-1 mRNA. Fig. 2C shows TIMP-1 mRNA levels in response to 500 μmol/l homocysteine (the concentration which elicited a maximal effect) after 8 and 24 h of incubation (Fig. 2C). We next tested whether homocysteine induction of TIMP-1 mRNA resulted in enhanced production of TIMP-1 protein. For this purpose we used the differentiated human hepatoma cell line HepG2, a hepatic cell line in which TIMP-1 expression and secretion has been thoroughly characterized in response to cytokines and phorbol esters [42]. HepG2 cells were incubated in the presence of increasing concentrations of homocysteine for 24 h in serum free DMEM, then conditioned media were collected and concentrated as described in Section 2. Samples were analyzed for the presence of TIMP-1 protein by Western blotting. As shown in Fig. 2D, homocysteine induced a dose-dependent accumulation of TIMP-1 protein in the medium of treated cells, as evidenced by the detection of a single 29-kDa band, the reported size of TIMP-1 protein [17]. This effect was already observed at 50 μmol/l homocysteine and was maximal at 250 μmol/l.

It has been suggested that some cellular effects of homocysteine could be mediated by its thiol group [13,14]. We have tested the effect of other thiol-containing reagents, such as cysteine and β-mercaptoethanol, on TIMP-1 expression. Treatment of HSCs for 4 h with these compounds (at concentrations of 500 μmol/l) resulted in the upregulation of TIMP-1 mRNA (Fig. 3). Interestingly, methionine treatment for the same period of time also promoted TIMP-1

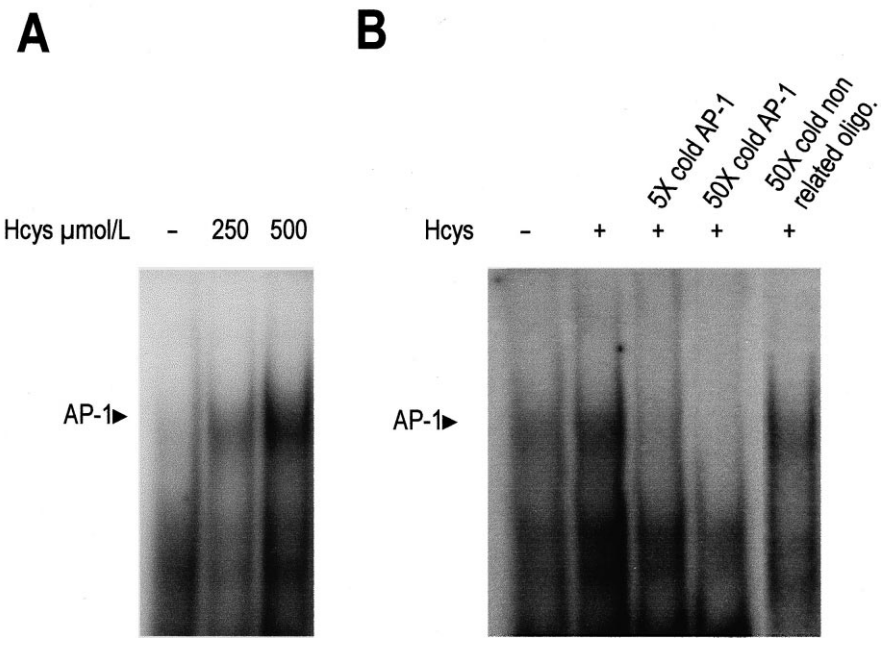

Fig. 4. Effect of homocysteine on DNA binding activity of AP-1 assayed by EMSA analysis. (A) Nuclear extracts from cultured rat hepatocytes treated for 2 h with different concentrations of homocysteine were incubated with ^{32}P-labeled AP-1 oligonucleotide encompassing the AP-1 consensus motif and analyzed by EMSA. The position of specific complexes is indicated. (B) 5- and 50-fold molar excess of specific unlabeled oligonucleotides were added to the binding reactions to show the specificity of the AP-1 complexes. Fifty-fold molar excess of a random oligonucleotide of the same size did not compete with the specific AP-1 complexes induced by homocysteine.

expression. It must be noted, however, that regular culture media already contain methionine and cysteine at concentrations of 300 µmol/l.

In the regulation of TIMP-1 gene expression, AP-1 elements present in its promoter have been shown to play a critical role in mediating the response to serum factors and other agonists, such as phorbol esters [43,44]. These observations, together with the ability of homocysteine to induce protein kinase C (PKC) and MAP kinase activation and c-fos expression [45,46], led us to examine if homocysteine could induce AP-1 binding activity. For this purpose rat hepatocytes were treated with different concentrations of homocysteine, then nuclear extracts were prepared and subjected to EMSA analysis using an oligonucleotide containing the consensus AP-1 sequence. Homocysteine treatment for 2 h of cultured rat hepatocytes resulted in the dose dependent induction of a specific bandshift (Fig. 4A), which could be competed by an excess of the same unlabeled oligonucleotide harboring the consensus AP-1 binding site and was not competed by a random oligonucleotide of the same length (Fig. 4B).

4. Discussion

Hyperhomocysteinemia is a condition in which plasma levels of homocysteine are transiently or persistently elevated. This situation may develop as a consequence of a variety of genetic backgrounds, such as deficiencies in cystathionine β-synthase or methylenetetrahydrofolate reductase, and pathological or nutritional conditions leading to vitamin B_6, B_{12} or folate deficiencies [1]. In addition, impaired liver function may lead as well to the elevation of plasma homocysteine. In this regard, hyperhomocysteinemia has been reported in chronic alcohol consumption with a homocysteine plasma concentration up to 40 µmol/l [6,7]. This homocysteine elevation also appears in experimental models of ethanol and CCl_4-induced liver damage (2.5- and 20-fold increase above basal levels of homocysteine respectively) [4,5], probably reflecting the central role played by this organ in homocysteine catabolism [9]. While the mechanisms that lead to an increased plasma homocysteine have been quite well established, the molecular basis of homocysteine-mediated alteration of

cellular function are not completely understood. In an effort to gain more insight into this issue we have searched for genes whose expression could be modulated by this amino acid. For this purpose we have carried out differential display analysis in VSMCs, a well characterized cellular target of homocysteine. In this cell type, homocysteine promotes DNA synthesis and cell growth, and enhances collagen production [12–15,45]. One of the homocysteine-respondent genes identified in our study coded for the TIMP-1 protein. TIMP-1 is a member of the tissue inhibitor of the metalloproteinases family, a secretory glycoprotein able to control the activity of MMPs through the direct interaction with these enzymes [17]. We have observed that homocysteine upregulates TIMP-1 mRNA levels in primary cultures of human and pig VSMCs. This novel action of homocysteine provides further information on the mechanisms by which this amino acid promotes net collagen deposition in the atherosclerotic lesion [11,47]. Given the central role that TIMP-1 plays in the regulation of ECM homeostasis we wanted to know whether this response to homocysteine would also take place in another scenario, such as liver tissue, in which matrix production has to be tightly controlled. Our results indicate that homocysteine is able to promote TIMP-1 mRNA expression in a HSCs clone and cultured hepatocytes, as well as the secretion of TIMP-1 protein by the hepatic cell line HepG2. Nevertheless, it would be interesting to determine whether TIMP-1 induction by homocysteine occurs in freshly isolated HSCs as it does in our HSC cell line, which in some aspects behave like activated HSCs. We have also observed that homocysteine promotes the expression of α1(I) procollagen mRNA in our HSCs clone. This later effect has been already reported for VSMCs in culture treated with homocysteine [13,14] and together with our present findings support a role for homocysteine in the regulation of ECM homeostasis in diverse tissular backgrounds.

Enhanced TIMP-1 production by HSCs and hepatocytes has been documented in experimental models of cholestatic and CCl_4-induced liver damage [22,48] and in patients with chronic liver disease [19,20,40]. Additionally, elevated plasma levels of TIMP-1 have been proposed as an early marker of fibrosis and precirrhotic states in humans [49]. As mentioned above, most of these situations of liver damage are accompanied by a rise in plasma homocysteine. However, to our knowledge, a direct role for this amino acid in the impairment of liver ECM regulation had not been so far established. Interestingly, the hepatic lesion observed in patients with homocystinuria, characterized by fatty infiltration, can be accompanied by perisinusoidal or central venous fibrosis and fibrosis of hepatic arterioles [50].

Previous studies have reported on the role of homocysteine thiol group in mediating many of the cellular effects of this amino acid [13–15]. We have tested whether other thiol-bearing compounds could have an effect on TIMP-1 mRNA expression. Treatment of HSCs with cysteine or β-mercaptoethanol resulted as well in the induction of TIMP-1 mRNA. Interestingly, methionine treatment gave a similar response. This might be explained by its rapid metabolic conversion into homocysteine [8,51,52]. These observations are in agreement with the previously reported induction of collagen synthesis by cysteine in VSMCs [14] and TIMP-1 expression in fibroblasts in response to reduced glutathione [53], and support the hypothesis of the implication of homocysteine sulfhydryl group in the mediation of some of the biological effects of this amino acid. Although the observed effect of cysteine on TIMP-1 induction may be of interest from a mechanistic point of view regarding a thiol-mediated effect, this effect is unlikely to have physiological or pathological significance. Such high cysteine concentrations are not observed even in patients with defects in cysteine metabolism [54], while the concentrations of homocysteine used in our experiments can be found in patients with intermediate and severe hyperhomocysteinemia [1].

The control of TIMP-1 expression, which mainly occurs at the transcriptional level, has been thoroughly characterized. The study of its promoter region has allowed the identification of the transcription factors critical for its regulation [43,44]. In this regard, AP-1 has been shown as one of the key factors that interact with TIMP-1 promoter and activate its transcription. This information, together with the recently described ability of homocysteine to promote PKC and MAP kinase activation and c-fos induction [45,46], prompted us to evaluate whether homocysteine treatment could result in an increased AP-1 binding activity. Our results indicate that this

is the case, as evidenced by the appearance of specific bandshifts for AP-1 in EMSA assays using nuclear extracts from rat hepatocytes treated with homocysteine. Our data agree with the previously reported induction of AP-1 by other thiol reagents, such as dithiocarbamates and *N*-acetylcysteine, which are able to promote c-fos and c-jun induction [55].

Taken together, our results indicate that homocysteine may contribute to the deposition of ECM by different, but converging mechanisms. In response to elevated levels of homocysteine cells such as VSMC and HSC respond producing more collagen. The concomitant induction of TIMP-1 in the same cell types and in neighboring hepatocytes would prevent its degradation favoring the net accumulation of ECM. In the vascular wall, this would result in the promotion of atherogenesis while in the liver tissue, the same situation may favor the progression of liver fibrosis. Furthermore, the elevated plasma levels of homocysteine, that accompany chronic alcohol consumption, genetic defects in its metabolism, and vitamin deficiencies, could be considered as a novel risk factor for liver disease. Homocysteine may thus cooperate in the onset of liver fibrosis under diverse pathological circumstances potentiating the effects of other agents, such as ethanol and cytokines [56].

Acknowledgements

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References

[1] H. Refsum, P.M. Ueland, O. Nygard, S.E. Vollset, Homocysteine and cardiovascular disease, Annu. Rev. Med. 49 (1998) 31–62.

[2] S.H. Mudd, H.L. Levy, F. Skovby, Disorders of transsulfuration, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 1995, pp. 693–734.

[3] J. Selhub, P.F. Jacques, P.W. Wilson, D. Rush, I.H. Rosenberg, Vitamin status and intake as primary determinants of homocysteinemia in an elderly populations, J. Am. Med. Assoc. 270 (1993) 2693–2698.

[4] G. Varela-Moreiras, E. Alonso-Aperte, M. Rubio, M. Gasso, R. Deulofeu, L. Alvarez, J. Caballeria, J. Rodes, J.M. Mato, Carbon tetrachloride-induced hepatic injury is associated with global DNA hypomethylation and homocysteinemia. Effect of S-adenosylmethionine treatment, Hepatology 22 (1995) 1310–1315.

[5] C.H. Halsted, J. Villanueva, C.J. Chandler, S.P. Stabler, R.H. Allen, L. Muskhelishvili, S.J. James, L. Poirier, Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation, Hepatology 23 (1996) 497–505.

[6] B. Hultberg, M. Berglund, A. Andersson, A. Frank, Elevated plasma homocysteine in alcoholics, Alcohol Clin. Exp. Res. 17 (1993) 687–689.

[7] M.L. Cravo, L.M. Gloria, J. Selhub, M.R. Nadeau, M.E. Camilo, M.P. Resende, J.N. Cardoso, C.N. Leitao, F.C. Mira, Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status, Am. J. Clin. Nutr. 63 (1996) 220–224.

[8] J.D. Finkelstein, J.J. Martin, Methionine metabolism in mammals: distribution of homocysteine between competitive pathways, J. Biol. Chem. 259 (1984) 9508–9513.

[9] J.D. Finkelstein, The metabolism of homocysteine: pathways and regulation, Eur. J. Pediatr. 157 (1998) S40–S44.

[10] J.S. Stamler, J.A. Osborne, O. Jakaki, L.E. Rabbani, M. Mullins, D. Singel, J. Loscalzo, Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen, J. Clin. Invest. 91 (1993) 308–318.

[11] S.R. Lentz, Homocysteine and vascular disfunction, Life Sci. 61 (1997) 1205–1215.

[12] J.C. Tsai, M.A. Perrella, M. Yoshizumi, C.M. Hsieh, E. Haber, R. Schlegel, M.E. Lee, Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis, Proc. Natl. Acad. Sci. USA 91 (1994) 6369–6373.

[13] J.C. Tsai, H. Wang, M.A. Perrella, M. Yoshizumi, N.E.S. Sibinga, L.C. Tan, E. Haber, T.H.T. Chang, R. Schlegel, M.E. Lee, Induction of cyclin A gene expression by homocysteine in vascular smooth muscle cells, J. Clin. Invest. 97 (1996) 146–153.

[14] A. Majors, L.A. Ehrhart, E.H. Pezacka, Homocysteine as a risk factor for vascular disease. Enhanced collagen production and accumulation by smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 2074–2081.

[15] S.C. Tyagi, Homocysteine redox receptor and regulation of extracellular matrix components in vascular cells, Am. J. Physiol. 274 (1998) C396–C405.

[16] O. Karmin, E.G. Lynn, Y.H. Chung, Y.L. Siow, R.Y.K. Man, P.C. Choy, Homocysteine stimulates the production

and secretion of cholesterol in hepatic cells, Biochim. Biophys. Acta 1393 (1998) 317–324.
[17] J.F. Woesner, Matrix metalloproteinases and their inhibitors in connective tissue remodeling, FASEB J. 5 (1991) 2145–2154.
[18] M.J.P. Arthur, Collagenases and liver fibrosis, J. Hepatol. 22 (1995) 43–48.
[19] H. Herbst, T. Wege, S. Milani, G. Pellegrini, H.D. Orzechowski, W.O. Bechstein, P. Neuhaus, A.M. Gressner, D. Schuppan, Tissue inhibitor of metalloproteinase-1 and -2 RNA expression in rat and human liver fibrosis, Am. J. Pathol. 150 (1997) 1647–1659.
[20] R.C. Benyon, J.P. Iredale, S. Goddard, P.J. Winwood, M.J.P. Arthur, Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver, Gastroenterology 110 (1996) 821–831.
[21] J.P. Iredale, C. Benyon, M.J.P. Arthur, W.F. Ferris, R. Alcolado, P.J. Winwood, N. Clark, G. Murphy, Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis, Hepatology 24 (1996) 176–184.
[22] E. Roeb, E. Purucker, B. Breuer, H. Nguyen, P.C. Heinrich, S. Rose-John, S. Matern, TIMP expression in toxic and cholestatic liver injury in rat, J. Hepatol. 27 (1997) 535–544.
[23] P. Greenwel, J. Rubin, M. Schwarzt, E.L. Hertzberg, M. Rojkind, Liver fat-storing cell clones obtained from CCl_4-cirrhotic rats are heterogeneous with regard to proliferation, expression of extracellular matrix components, interleukin-6 and connexin 43, Lab. Invest. 69 (1993) 210–216.
[24] N.F. González-Cadavid, D. Vernet, A. Fuentes, J.A. Rodríguez, R. Swerdloff, J. Rajfer, Up-regulation of the levels of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis, Mol. Cell. Endocrinol. 90 (1993) 219–229.
[25] M.A. Avila, M.V. Carretero, E.N. Rodriguez, J.M. Mato, Regulation by hypoxia of methionine adenosyltransferase activity and gene expression in rat hepatocytes, Gastroenterology 114 (1998) 364–371.
[26] G. Otero, M.A. Avila, L. de la Peña, D. Emfietzoglou, J. Cansado, G.F. Popescu, V. Notario, Altered processing of precursor transcripts and increased levels of the subunit I of mitochondrial cytochrome c oxidase in syrian hamster fetal cells initiated with ionizing radiation, Carcinogenesis 18 (1997) 1569–1575.
[27] P. Liang, A.B. Pardee, Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967–971.
[28] G.H. Hamon, G.N. Cameron, The EMBL data library, Nucleic Acids Res. 14 (1986) 5–10.
[29] H.S. Bilofsky, C. Burks, The GeneBank data bank, Nucleic Acids Res. 16 (1988) 1861–1864.
[30] W.R. Pearson, D.J. Lipmann, Improved tools for biological sequence comparison, Proc. Natl. Acad. Sci. USA 85 (1988) 2444–2448.
[31] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[32] P.S. Thomas, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose, Proc. Natl. Acad. Sci. USA 77 (1980) 5201–5205.
[33] G. Genovese, D. Rowe, B. Kream, Construction of DNA squences complementary to rat alpha 1 and alpha 2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D, Biochemistry 23 (1984) 6210–6216.
[34] E. Schreiber, P. Matthias, M.M. Muller, W. Schaffner, Rapid detection of octamers binding proteins with miniextracts prepared from a small number of cells, Nucleic Acids Res. 17 (1989) 6419.
[35] A.J.P. Docherty, A. Lyons, B.J. Smith, E.M. Wright, P.E. Stephens, T.J.R. Harris, Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity, Nature 318 (1985) 66–69.
[36] C.M. Overall, J.L. Wrana, J. Sudek, Independent regulation of collagenase72 KD progelatinase, and metalloendoproteinase inhibitor expression in human fibroblast by transforming growth factor-β, J. Biol. Chem. 264 (1989) 1860–1869.
[37] D.R. Edwards, G. Murphy, J.J. Reynolds, S.E. Whitham, A.J. Docherty, P. Angel, J.K. Heath, Transforming growth factor beta modulates expression of collagenase and metalloproteinase inhibitor, EMBO J. 6 (1987) 1899–1904.
[38] S.L. Friedman, The cellular basis of hepatic fibrosis, New Engl. J. Med. 328 (1993) 1828–1835.
[39] J.P. Iredale, G. Murphy, R.M. Hembry, S.L. Friedman, M.J.P. Arthur, Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinases-1, J. Clin. Invest. 90 (1992) 282–287.
[40] Y. Murawaki, Y. Ikuta, Y. Idobe, Y. Kitamura, H. Kawasaki, Tissue inhibitor of metalloproteinase-1 in the liver of patients with chronic liver disease, J. Hepatol. 26 (1997) 1213–1219.
[41] E. Roeb, L. Graeve, R. Hoffmann, K. Decker, D.R. Edwards, P.C. Heinrich, Regulation of tissue inhibitor of metalloproteinases-1 gene expression by cytokines and dexametasone in rat hepatocytes primary cultures, Hepatology 18 (1993) 1437–1442.
[42] T. Kordula, I. Güttgemann, S. Rose-John, E. Roeb, A. Osthues, H. Tschesche, A. Koj, P.C. Heinrich, L. Graeve, Synthesis of tissue inhibitor of metalloproteinase-1 (TIMP-1) in human hepatoma cells (HepG2), FEBS Lett. 313 (1992) 143–147.
[43] S.K. Logan, M.J. Garabedian, C.E. Campbell, Z. Werb, Synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors, J. Biol. Chem. 271 (1996) 774–782.
[44] F.M. Botelho, D.R. Edwards, C.D. Richards, Oncostatin stimulates c-fos to bind a transcriptionally responsive AP-1 element within the tissue inhibitor of metalloproteinase-1 promoter, J. Biol. Chem. 273 (1998) 5211–5218.

[45] M.L. Dalton, P.F. Gadson, R.W. Wrenn, T.H. Rosenquist, Homocysteine signal cascade: production of phospholipids, activation of protein kinase C, and the induction of c-fos and c-myb in smooth muscle cells, FASEB J. 11 (1997) 703–711.
[46] J.C. Brown III, T.H. Rosenquist, D.T. Monaghan, ERK2 activation by homocysteine in vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 251 (1998) 669–676.
[47] K.S. Mc Cully, Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis, Am. J. Pathol. 56 (1969) 111–121.
[48] A.E. Kossakowska, D.R. Edwards, S.S. Lee, L.S. Urbanski, A.L. Stabbler, C.L. Zhang, B.W. Phillips, Y. Zhang, S.J. Urbanski, Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis, Am. J. Pathol. 153 (1998) 1895–1902.
[49] J. Li, A.S. Rosman, M.A. Leo, Y. Nagai, C.S. Lieber, Tissue inhibitor of metalloproteinase is increased in the serum of precirrhotic and cirrhotic alcoholic patients and can serve as a marker of fibrosis, Hepatology 19 (1994) 1418–1423.
[50] G. Klastin, H.O. Conn, Hepatic lesions in disorders of protein and amino acid metabolism, in: Histopathology of the Liver, Oxford University Press, New York, 1993, pp. 241–245.
[51] M.F. Bellamy, I.F. McDowell, M.W. Ramsey, M. Brownlee, C. Bones, R.G. Newcombe, M.J. Lewis, Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults, Circulation 98 (1998) 1848–1852.
[52] P. Durand, S. Lussier-Cacan, D. Blache, Acute methionine load-induced hyperhomocysteinemia enhances platelet aggregation, thromboxane biosynthesis, and macrophage-derived tissue factor activity in rats, FASEB J. 11 (1997) 1157–1168.
[53] S.C. Tyagi, G.S. Kumar, S. Borders, Reduction-oxidation (redox) state regulation of extracellular matrix metalloproteinases and tissue inhibitors in cardiac normal and transformed fibroblast cells, J. Cell. Biochem. 61 (1996) 139–151.
[54] S. Segal, S.O. Thier, Cystinuria, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, (Eds.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 1995, pp. 3581-3601.
[55] M. Meyer, R. Schreck, P.A. Bauerle, H_2O_2 and antioxidants have opposite effects on activation of NF-kB and AP-1 in intact cells: AP-1 as a secondary antioxidant-responsive factor, EMBO J. 12 (1993) 2005–2015.
[56] S. Degli Esposti, M.A. Zern, Peptide hormone and cytokine regulation of matrix synthesis, in: M.A. Zern, L.M. Reid (Eds.), Extracellular matrix, Marcel Dekker, New York, 1993, pp. 331-349.